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Effect of pH on the catalytic function and zinc content of native and immobilized anthrax lethal factor

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ABSTRACT

Translocation of the zinc-dependent metalloendopeptidase anthrax lethal factor (LF) from the endosome to the cytosol requires an acidic endosomal milieu. In the current study, we utilized immobilized (to prevent protein aggregation below pH 5.5) and native LF to assess the effect of pH on the function and metal content of LF. Our results reveal the diminution of LF's catalytic competence under moderately acidic conditions (pH ~6) to be uncorrelated to the metal content of the protein. However, a significant degree of demetallation of LF (~30%) was observed at pH values close to those found in late endosomes (pH ~5), thus raising the possibility that a substantial proportion of LF molecules may not be in their zinc-bound state prior to translocation.

Structured summary of protein interactions:
LF and **LF** bind by fluorescence technology (View interaction)

1. Introduction

Anthrax toxin (AT), an exotoxin secreted by *Bacillus anthracis* and a major virulence factor of anthrax, is a tripartite protein comprised of protective antigen (PA), edema factor (EF) and lethal factor (LF) [1]. While EF acts as an adenylyl cyclase responsible for the increase in intracellular cAMP levels [2], LF is a zinc-dependent metalloendopeptidase involved in the cleavage of mitogen-activated protein kinase kinases near their N-termini [3–6], and thus in the impairment of critical intracellular signaling pathways [7]. In addition, LF has recently been implicated in catalyzing the removal of the N-terminal segment of NOD-like receptor protein 1 (Nlrp1), leading to inflammasome activation and macrophage death [8]. PA (in its oligomeric form) is a pore-forming protein which mediates the entry of LF and EF from the endosome into the host cell cytosol [9–11]. In view of the dimensions of the central lumen of the PA pore (~15 Å diameter at its narrowest point), it has been proposed that structural arrangements only as large as a single α-helix are capable of passing through the pore [12,13]. Consequently, PA-assisted translocation of LF and EF requires the (at least) partial unfolding of the enzymatic components [12–15]. In addition, an acidic milieu, imposed by the vacuolar H^+-ATPases in the endosomal membrane [16], is stringently required to not only trigger pore formation, but also to assist in the initiation of unfolding of the N-terminus of LF (or EF) [15]. Given that the acidification of zinc proteins can render the metal ion prone to dissociation (by virtue of protonation of the metal-binding ligands [17]), it is not inconceivable that LF's Zn^{2+} ion (being bound to His686, His690 and Glu735) is released at low endosomal pH. However, whether LF's Zn^{2+}-binding motif can stay intact under these conditions remains to be established.

Previous studies on the pH dependence of LF's catalytic competence have revealed the enzymatic activity of the protein to be significantly reduced below pH 6, and to be almost completely abolished at pH 4 [18,19]. Although the molecular basis underlying these observations has not been elucidated, loss of the catalytically essential metal ion might provide a plausible explanation for this phenomenon.

The current investigations were aimed at elucidating the relationship between LF's catalytic function and its metal status at pH values encountered within the endosome. It is important to note that preliminary studies on the influence of pH on LF revealed the protein to precipitate below pH 5.5, a feature which could potentially affect both the pH-dependence of catalytic function and the enzyme's ability to bind metal ions. To avoid aggregation,

Abbreviations: AMT, acetic acid/MES/Tris; AT, anthrax toxin; cAMP, adenosine 3′,5′-cyclic monophosphate; EF, edema factor; I_c, ionic strength (concentrational); $LF_{(nat)}$, (native) lethal factor; LF_{im}, immobilized lethal factor; MES, 2-(*N*-morpholino)ethanesulfonic acid; NHS, *N*-hydroxysuccinimide; PA, protective antigen; *S*-*p*NA, lethal factor substrate; Tris, tris(hydroxymethyl)aminomethane

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LF was immobilized onto *N*-hydroxysuccinimide (NHS) activated agarose beads (see Fig. 1), and its catalytic competence and metal content was assessed as a function of pH, with the non-immobilized, native form of the protein serving as a reference.

2. Materials and methods

2.1. General

Lethal factor protease substrate II (*S*-*p*NA) was purchased from EMD Biosciences (La Jolla, CA). All other chemicals were of analytical grade and obtained from Sigma–Aldrich (St. Louis, MO). All solutions were prepared using MilliQ ultrapure water ($\geqslant$18.2 MΩ cm resistivity). Unless stated otherwise, AMT, a three-component buffer consisting of acetic acid, 2-(*N*-morpholino)ethanesulfonic acid (MES) and tris(hydroxymethyl)aminomethane (Tris) in a 1:1:2 molar ratio was utilized in the current studies to maintain a constant ionic strength (I_c) over the entire pH range investigated [20]. The pH of the AMT buffer was adjusted to the desired value using either HCl or NaOH.

2.2. Isolation and immobilization of LF

LF was isolated from *Bacillus megaterium* harboring the pWH1520-LF plasmid (MoBiTec, Göttingen, Germany) and purified as described previously [21]. The Zn^{2+} content of purified LF was determined with 4-(2-pyridylazo)resorcinol as outlined earlier [22], and was found to be 1.0 (±0.1) Zn^{2+} per protein molecule. Immobilization of LF was achieved by covalently coupling the protein to *N*-hydroxysuccinimide (NHS)-activated agarose beads (Pierce, Rockford, IL) according to the manufacturer's instructions, using spin columns containing either 33 or 330 mg of resin. In brief, LF was exchanged into sodium phosphate buffer (100 mM, pH 7.2) containing 0.15 M NaCl prior to coupling. Immobilization of LF was achieved by incubating the protein (10 μM) in sodium phosphate buffer in the presence of the beads for 1 h at room temperature. Following coupling, the mixture was centrifuged at 1000×*g* for 1 min, and the flow-through was kept for the determination of the protein coupling efficiency (see below). The beads were then washed twice with sodium phosphate buffer. Any unreacted (NHS-containing) sites were subsequently blocked by treatment with ethanolamine (1 M, pH 7.4) for 30 min at room temperature. Following centrifugation, the beads were washed three times with AMT buffer (12.5 mM acetic acid, 12.5 mM MES and 25 mM Tris; I_c = 0.025; pH 7.0) to remove excess ethanolamine. The protein coupling efficiency was determined using the Pierce 660 nm protein assay according to manufacturer's instructions.

2.3. Determination of enzymatic activity

To maintain a constant ionic strength over the pH range investigated (pH 4.0–8.0), AMT buffer (I_c = 0.025) was utilized as the medium in the assessment of the catalytic competence of LF. The choice of the ionic strength (I_c = 0.025) was based on the observation that LF is stable and catalytically fully functional under these conditions [19]. Following incubation of the protein at the desired pH for 3 min at room temperature, the enzymatic activity of the native form of LF (LF_{nat}) was determined spectrophotometrically at 405 nm using *S*-*p*NA as reported in the literature [22,23]. In the case of immobilized LF (LF_{im}), the protocol for the determination of the enzyme's catalytic competence required significant alterations in view of increased light scattering at 405 nm, and the occurrence of incomplete mixing of substrate and enzyme as a consequence of the agarose beads settling rapidly to the bottom of the spectrophotometer cell. Hence, the activity of LF_{im} was assessed by exposing the protein (100 nM) to AMT buffer (I_c = 0.025) of the desired pH for 3 min at room temperature prior to the initiation of the reaction by the addition of *S*-*p*NA (10 μM). Following exposure to the substrate for 120 s, LF_{im} was removed rapidly (within 5 s) by passing the mixture through an empty spin column. The concentration of the reaction product (*p*-nitroaniline) was determined at 405 nm using an extinction coefficient of 9920 M^{-1} cm^{-1} [23].

2.4. Assessment of zinc release from LF

In the case of LF_{nat}, a stock solution of the enzyme (35 μM) was diluted with AMT buffer (I_c = 0.0188) of the desired pH to achieve a final concentration of 0.6 μM with respect to the enzyme. Following incubation for 3 min at room temperature, the protein was removed by centrifugation using an Amicon Ultra centrifugal concentrator (30 kDa molecular weight cut-off; Millipore, Bedford, MA), and the recovered filtrate was diluted threefold with MilliQ water prior to metal determination. In case of complete release of Zn^{2+} from LF, the expected concentration of the ion in the filtrate was 0.2 μM (13.1 ppb).

To assess the pH-dependence of Zn^{2+} dissociation from LF_{im}, 100 μl of the enzyme ([LF_{im}] = 6.8 μM in AMT buffer [I_c = 0.025, pH = 7.0]) was supplemented with an equal volume of AMT buffer (I_c = 0.1) to achieve the desired pH. Following incubation for 3 min at room temperature, the mixture was passed through an empty spin column to remove the immobilized protein. The filtrate was subsequently diluted 10-fold with MilliQ water so as to achieve a final ionic strength of AMT buffer identical to that outlined above for LF_{nat} (i.e., I_c = 0.00625). In case of complete release of Zn^{2+} from LF, the anticipated concentration of the ion in the filtrate was 0.34 μM (22.2 ppb).

The amount of Zn^{2+} in the filtrates was determined by inductively-coupled plasma mass spectrometry using a Perkin Elmer Sciex Elan 6000 mass spectrometer (Waltham, MA). External Zn^{2+} standards (1–40 ppb) were prepared in AMT buffer of the same ionic strength as that used for protein samples (i.e., I_c = 0.00625). All samples (including standards) were supplemented with HNO_3 (trace metal grade) to achieve a final concentration of 1% (w/v) prior to measurement (in triplicate) using standard operating conditions.

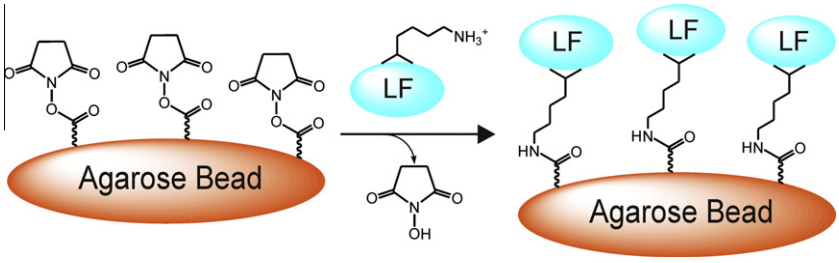

Fig. 1. Schematic representation of the immobilization of LF using NHS-activated agarose beads.

2.5. Spectroscopy

The degree of LF aggregation at low pH was assessed by recording the optical density of the protein solution ([LF] = 5 μM) at 330 nm ($OD_{330\ nm}$) using a Biochrom Ultrospec 2100 *pro* spectrophotometer (Cambridge, UK). Fluorescence emission spectra of LF (0.5 μM) were recorded with the aid of an OLIS RSM1000 spectrofluorometer (Bogart, GA) equipped with a 150 W Xenon arc lamp. The excitation wavelength was set to 295 nm to allow for the selective excitation of LF's five tryptophan residues.

3. Results

3.1. Aggregation of LF at low pH

Preliminary studies on the influence of pH on (micromolar levels of) LF revealed the protein to form visible aggregates in solution under mildly acidic conditions. As shown in Fig. 2, the degree of light scattering at 330 nm, a wavelength at which the aromatic amino acid residues of LF do not absorb, increased markedly upon lowering the pH from 5.3 to 5.2. It is important to note that the onset of LF aggregation coincides reasonably well with the protein's theoretical pI value of 5.5 (calculated based on the primary sequence of LF). Thus, it appears likely that precipitation of LF at low pH is a consequence of the protein becoming electrically neutral. To assess whether LF forms aggregates at sub-micromolar levels (i.e., at concentrations relevant to activity and metal content determinations), the pH dependence of the enzyme's intrinsic tryptophan fluorescence emission spectra was recorded. As shown in Fig. 2 (inset), the λ_{max} value of the emission spectra at 333 nm was found to remain constant from pH 4.0 to 7.0, indicating that the C-terminal domains of LF (which house the protein's five tryptophan residues [3]) remain folded upon acidification. However, the significant increase in fluorescence intensity at 300 nm between pH 4 and 5 is a clear reflection of increased scattering, and thus of protein aggregation.

3.2. Immobilization of LF

To prevent protein aggregation at low pH, a feature which could potentially alter the protein's pH dependence of catalytic function and metal content, LF was immobilized on NHS-activated agarose beads. Indeed, the attachment of LF to a solid support is reminiscent of the situation prevailing in vivo, where following endocytosis, LF molecules are spatially separated in view of their attachment to the PA prepore [15,24]. Immobilization of LF to the beads was achieved with a coupling efficiency of 97 (±2)%, and the enzyme's metal content was found to be 1.0 (±0.1) Zn^{2+}/LF, suggesting that coupling to a solid support did not impair Zn^{2+} binding. To assess whether aggregation of LF, a phenomenon observable with the non-immobilized, native form of the protein (LF_{nat}) at low pH, affects the enzyme's catalytic function and metal status, pH studies were performed in a comparative manner employing both immobilized LF (LF_{im}) and LF_{nat}.

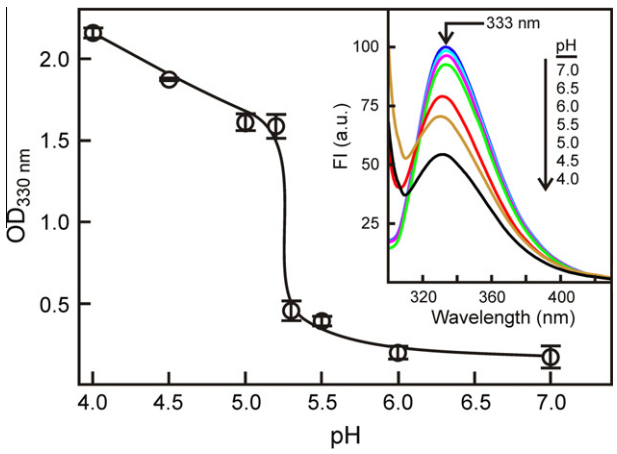

Fig. 2. Aggregation of LF. The degree of LF aggregation was assessed at 330 nm using LF_{nat} (5 μM) in AMT buffer (I_c = 0.025) of the desired pH. Values shown denote the mean of three independent experiments (±1 S.D.). Inset: Intrinsic tryptophan fluorescence emission spectra of LF. LF (0.5 μM) was incubated in AMT buffer of the pH indicated in the figure for 5 min at room temperature prior to recording emission spectra. FI (a.u.) denotes the fluorescence intensity in arbitrary units.

3.3. pH dependence of catalytic activity

As shown in Fig. 3A, the pH profile of LF_{nat} revealed the loss of activity upon acidification to be biphasic. Between pH 6.75 and 8.0, maximal activity of LF was achieved. Below pH 6.75, the activity of LF decreased to approximately 60% of that recorded at pH 7.0, with no further diminution of catalytic competence noted upon further acidification to pH 5.75. At pH values below 5.75, a progressive loss of activity of LF was observed, with the protein being virtually inactive at pH 4.0. At first glance, the pH profile of LF_{im} (Fig. 3B) appears to be similar to that recorded for LF_{nat}, especially with respect to the biphasic nature of the titration and the

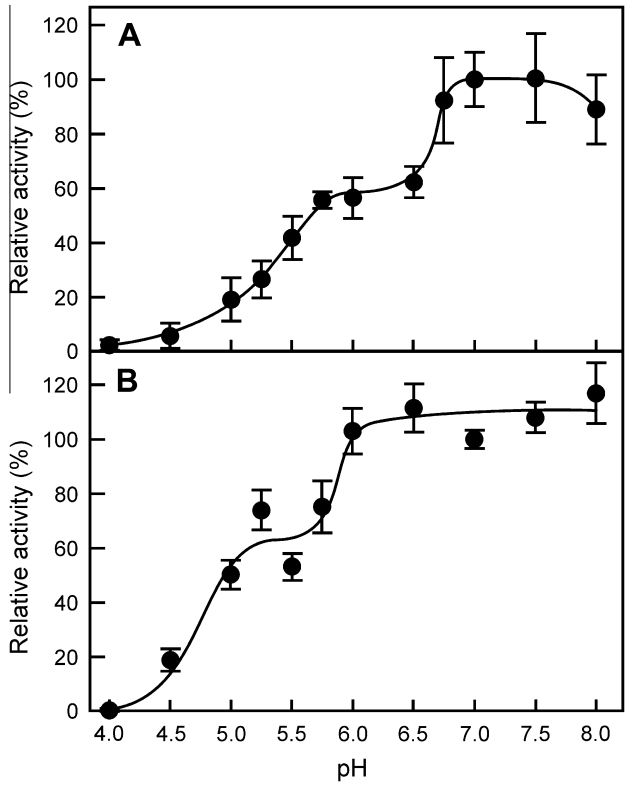

Fig. 3. pH-dependence of catalytic activity of LF_{nat} (A) and LF_{im} (B). The activity of LF_{nat} was assessed by incubating the protein (50 nM) in AMT buffer (I_c = 0.025) of the desired pH for 3 min at room temperature prior to initiation of the assay by introduction of *S*-*p*NA (10 μM). Activities are expressed relative to that observed at pH 7.0, and are depicted as the mean (±1 S.D.) of three independent experiments. The activity of LF_{im} was assessed by incubating the protein (100 nM) in AMT buffer (I_c = 0.025) of the desired pH for 3 min at room temperature prior to the addition of *S*-*p*NA (10 μM). Following 120 s of exposure to the substrate, the enzyme was removed and the concentration of product formed during the reaction was determined spectrophotometrically at 405 nm. Relative activities (ratio of product formed at the indicated pH and pH 7.0) are shown as the average (±1 S.D.) of three independent experiments.

complete loss of function at pH 4. However, in contrast to LF_{nat}, maximal activity of LF_{im} was maintained as low as pH 6.0. In addition, the activity plateau observed with LF_{im} (albeit also at ~60%) was found to be shifted towards lower pH values (between pH 5.0 and 5.75, instead of between pH 5.75 and 6.5 in the case of LF_{nat}). Furthermore, the activities of LF_{im} at pH 4.5 and 5.0 were significantly higher than those observed for LF_{nat}.

3.4. pH dependence of metal dissociation

While LF_{nat} was capable of retaining its Zn^{2+} ion upon acidification up to a pH value of 5.5, a significant increase in the amount of Zn^{2+} released from the enzyme (~40%) was observed at pH 5.25 (Fig. 4A). It is interesting to note that the pH value at which this marked loss of Zn^{2+} occurred coincides with that recorded for the onset of LF aggregation (see Fig. 2). Between pH 5.25 and 4.5, no further diminution of the metal content of LF was observed, whereas below pH 4.5, a sharp increase in the amount of Zn^{2+} released was noted, with the protein being virtually devoid of the metal ion at pH 4.0.

Regarding the release of Zn^{2+} from LF_{im}, Fig. 4B shows that similar to LF_{nat}, LF_{im} was found to be prone to Zn^{2+} dissociation between pH 4.0 and 4.5, and was capable of binding its full complement of metal between pH 5.5 and 7.0. However, the pH titration profiles of LF_{im} and LF_{nat} differ significantly between pH 4.5 and 5.5, with a more gradual release of Zn^{2+} (absence of a plateau) over this pH range in the case of the former protein preparation.

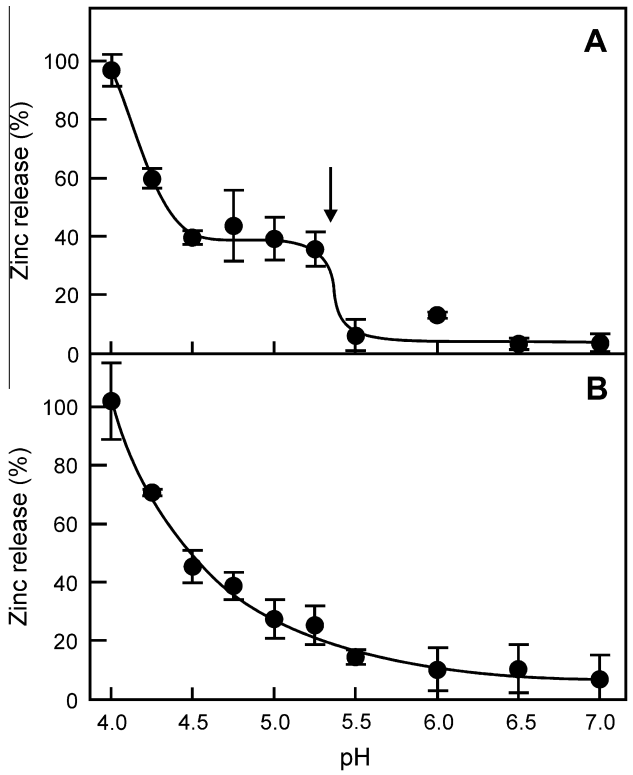

Fig. 4. pH-dependent release of Zn^{2+} from LF_{nat} (A) and LF_{im} (B). The amount of Zn^{2+} released from LF as a function of pH was assessed by ICP-MS following incubation of LF_{nat} and LF_{im} in AMT buffer of the desired pH for 3 min at room temperature, and subsequent removal of the protein. Values are expressed relative to that anticipated for the complete release of Zn^{2+} from the enzyme (0.20 μM for LF_{nat}, 0.34 μM for LF_{im}; see Section 2). Error bars represent ±1 S.D. of three independent experiments. The arrow in panel A indicates the pH at which aggregation of LF occurs.

4. Discussion

The impairment of catalytic function of metalloenzymes upon acidification is typically due to the protonation of catalytically relevant entities in or near the active site, the loss of structural integrity, or the release of the metal as a consequence of ligand protonation. Previous investigations on native LF have revealed the protein to lose activity at pH ⩽ 6, and to be virtually inactive at pH 4 [18,19]. Furthermore, the propensity of LF to become non-cytotoxic and to partially denature at pH 5.5 has been documented [25]. The observations recorded herein are in general accordance with these reports, although the biphasic nature of the decrease in enzyme activity upon acidification was observed only in the current study, presumably due to the larger amount of pH values investigated. The molecular origin underlying the difference in the pH profiles of LF_{nat} and LF_{im}, especially with respect to the activity at pH 6 (which is comparable to that at pH 7 in the latter case only) remains to be established. However, it is interesting to note that, similar to LF_{im}, the catalytic efficiency of thermolysin, a close relative of LF with respect to its active site architecture, remains unaltered upon acidification from pH 7 to 6 [26,27]. The impairment of thermolysin's catalytic function below pH 6 has generally been attributed to the protonation of the Glu_{143}–COO^-–H_2O–Zn^{2+} linkage [28]. Thus, it is not unlikely that the loss of catalytic function of LF_{im} below pH 6 (and of LF_{nat} at pH ⩾ 6) originates from protonation of such linkage involving the enzyme's Glu687 residue.

Since the PA-mediated translocation of LF proceeds from an acidified endosome, a few comments regarding its ionic content and pH are warranted. The ionic strength of (late) endosomes, which is mainly governed by the concentrations of chloride, sodium and potassium ions, is believed to be approximately 0.05, and thus similar to that employed in the current study [29]. The endosomal pH depends on the cell type and the maturity of this cellular organelle, but is generally considered to be between pH 4.8 and 6 [30,31]. Since the coordination of a zinc ion lowers the pK_a value(s) of its associated ligand(s) by (only) about 2–3 log units [32,33] (e.g., from 7.0 to 4.5 for Zn^{2+} binding to imidazole [34]), it is not inconceivable that a significant proportion of LF molecules are prone to releasing Zn^{2+} at pH ⩽ 5 (i.e., at the lower pH boundary of late endosomes). Indeed, the observations documented in the current study suggest that approximately 50% of Zn^{2+} dissociates from LF_{im} and LF_{nat} at a pH near 4.5. Interestingly, the significant increase in the release of Zn^{2+} upon acidification from pH 5.5 to 5.25, observed only with LF_{nat}, was found to coincide with the pH at which protein aggregation occurs. It is therefore tempting to speculate that the sudden onset of Zn^{2+} dissociation in this pH range is a consequence of structural changes imposed by the formation of protein aggregates (not observable with LF_{im}), rather than being due to the mere protonation of Zn^{2+} ligands.

The current report demonstrates that the loss of catalytic function of LF under moderately acidic conditions (pH ~6) does not correlate with the release of Zn^{2+} from the enzyme, which occurs at much lower pH. Although the majority of LF is capable of retaining its Zn^{2+} at low pH, a significant proportion of LF molecules would appear to be demetallated, especially near the lower pH boundary of late endosomes. As previously mentioned, it is currently unknown whether LF's Zn^{2+} ion is co-translocated along with the protein in vivo. The Zn^{2+} ion in the active site of LF is bound to both His686 and His690 from the 4α4 helix, and to Glu735 from the 4α6 helix [3]. If, indeed, the PA pore were to allow the passage of structures only as large as one individual α-helix (as has been suggested [12,13]), then LF's Zn^{2+}-binding motif would undoubtedly be disrupted when passing through the narrow lumen of the pore. However, it is not inconceivable that the metal ion is guided

through the pore by remaining bound to His686 and His690 located on the 4α4 helix. On the other hand, if Zn^{2+} were to be lost directly prior to or during LF translocation (be it as a consequence of acid-induced demetallation, a feature demonstrated in the current in vitro study, or the pH-driven PA-mediated partial unfolding of LF, or a combination of both), reactivation of the protein would require delivery of the metal ion from the cytosolic free Zn^{2+} pool, a non-trivial process given the very low concentrations of intracellular free Zn^{2+} in eukaryotic cells [35]. In such event, the high (picomolar) affinity of apoLF for Zn^{2+} [22] might be ultimately responsible for reestablishing the catalytic competence and, hence, the toxic effect(s) of LF in the cytosolic milieu of targeted host cells. It needs to be emphasized that, at present, any of the aforementioned proposals fall into the realm of conjecture due to the lack of experimental support. Nonetheless, the current report demonstrates, for the first time, the propensity of Zn^{2+} to dissociate from LF at pH values close to those found within late endosomes, and may thus provide an impetus to future investigations regarding the fate of the protein's metal ion during PA-mediated translocation.

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